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THE INTERMOLT CYCLE OF CIRRIPEDS: CRITERIA FOR ITS STAGES AND ITS DURATION IN *BALANUS AMPHITRITE*

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Many physiological and behavioral responses in crustaceans change markedly during the course of an intermolt cycle. To take such variations into account it is necessary to identify the stages of the intermolt cycle. Drach (1939, 1944) successfully divided the intermolt cycle of brachyurans and natantians into four main stages, A, B, C, and D, and, using structural changes in the integument, developed criteria by which these stages could be identified. Drach's method has found wide application within the Malacostraca (see Yamaoka and Scheer, 1970). In the Cirripedia, however, an intermolt staging method for live animals chosen at random has not been available. Studies which have related to the intermolt cycle in cirripeds have generally used animals at timed intervals after ecdysis (Patel and Crisp, 1961; Barnes and Barnes, 1963; Costlow, 1963; Shimony and Nigrelli, 1972). A requisite for the successful application of the timed interval method is intermolt cycles of uniform duration. This condition, however, may not be met in the cirripeds. Costlow and Bookhout (1953, 1956) found wide variations in the durations of the intermolt cycles of juvenile specimens of *Balanus improvisus* and *B. amphitrite;* there is no information in this regard for adult barnacles.

Intermolt stages in cirripeds have been morphologically identified in only a few studies. Using the criteria of Drach, Bocquet (1956) identified Stages D_1 and D_2 in *Chthamalus stellatus* by microscopically examining the cirri. Bocquet-Vedrine (1965) developed a method for histologically identifying intermolt stages of *Elminius modestus* in thin sections of whole animals. The present study had two major objectives: (1) to develop an intermolt staging method for barnacles which can utilize live, intact specimens taken at random, and (2) to statistically analyze the intermolt cycle of adult specimens of *B. amphitrite*.

Materials and Methods

Specimens of the intertidal barnacle *Balanus amphitrite amphitrite* Darwin (Harding, 1962) were collected from the noncreosoted portions of the laboratory dock in Beaufort, North Carolina, from February to November, 1972. The rostrocarinal diameter of the animals ranged from 5 to 10 mm. The animals were maintained in aquaria containing continuously aerated seawater at a salinity of 30‰ and fed *Artemia* nauplii (Metaframe San Francisco Bay Brand) for one month prior to experimentation. During the experiments the animals were maintained individually in compartmentalized plastic boxes in 40 to 50 ml of seawater at a

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salinity of 30‰. The boxes were kept in culture cabinets at 23° C with a LD 12:12 photoperiod. The seawater was changed and the animals fed 24 hr old *Artemia* nauplii daily. The compartments were checked for exuviae twice daily. Ecdysis was defined as having occurred midway between the observation when an exuvium was discovered and the previous observation. Animals which produced a brood during the experimental period, or which had ovigerous lamellae at the end of the period were discarded.

For the development of criteria for the intermolt stages rami were snipped off of each animal successively during the course of one to three intermolt cycles at approximately one day intervals. Rami were obtained with a pair of fine forceps, snipping while the animals were pumping or feeding. Only rami from the 4th, 5th, and 6th cirri were used. For microscopic examination the rami were mounted on slides in either seawater or glycerin jelly. For the latter method a number of slides were prepared beforehand by placing a drop of molten glycerin jelly on each slide and allowing it to solidify. The rami were placed in a drop of seawater atop the glycerin jelly, a coverslip was added, and the slide was placed on a slide warmer (48 to 50° C) just long enough to liquefy the glycerin jelly. It was necessary to examine the rami in both seawater and glycerin jelly. In seawater the ramial tissues have high contrast and are easily seen, however, it is difficult to resolve the exoskeletal layers because of extensive refraction. Glycerin jelly reduces the amount of refraction and thus improves the resolution of the exoskeleton, but at the same time obscures the other ramial structures. Observations and measurements of the exoskeletal layers were accordingly made on glycerin jelly mounted rami while observations of the other ramial structures were made on seawater mounted rami.

Measurements of the exoskeletal layers were made with a filar occular micrometer (American Optical) in the middle portion of the posterior side of the segment. Unless otherwise stated all measurements were made on the 13th segment from the tip of the ramus. Repeated measurements showed that the method was reproducible within ±5 per cent. The accuracy of the measurements and the visible presence of the exoskeletal layers in the whole mounts were checked further by making parallel measurements on whole mounted rami and sectioned rami: The bodies of eight animals were dissected free of their shells and one ramus from each was mounted in glycerin jelly. The remainder of each of the bodies was fixed in Helly's fluid, dehydrated in a graded series of ethanol, cleared in xylene, and embedded in *Paraplast*. Serial sections were cut at 7 to 8 μ and stained according to Hubschman's (1962) modification of Gomori's Azan method. In the whole mounts the posterior-anterior diameter and the thickness of the procuticle and of the exocuticle were measured. In the sections the thicknesses of the procuticle and exocuticle were measured at a point in a ramus where the diameter was the same as that in the corresponding whole mounted ramus. The measurements of the whole mounts agreed well with those of the sectioned material: The correlation coefficients for the measurements of the procuticle and exocuticle were 0.85 and 0.97, respectively.

Microscopic examinations and photomicrographs were made with a Leitz Labolux microscope. Statistical analyses were made according to Sokal and Rohlf (1969).

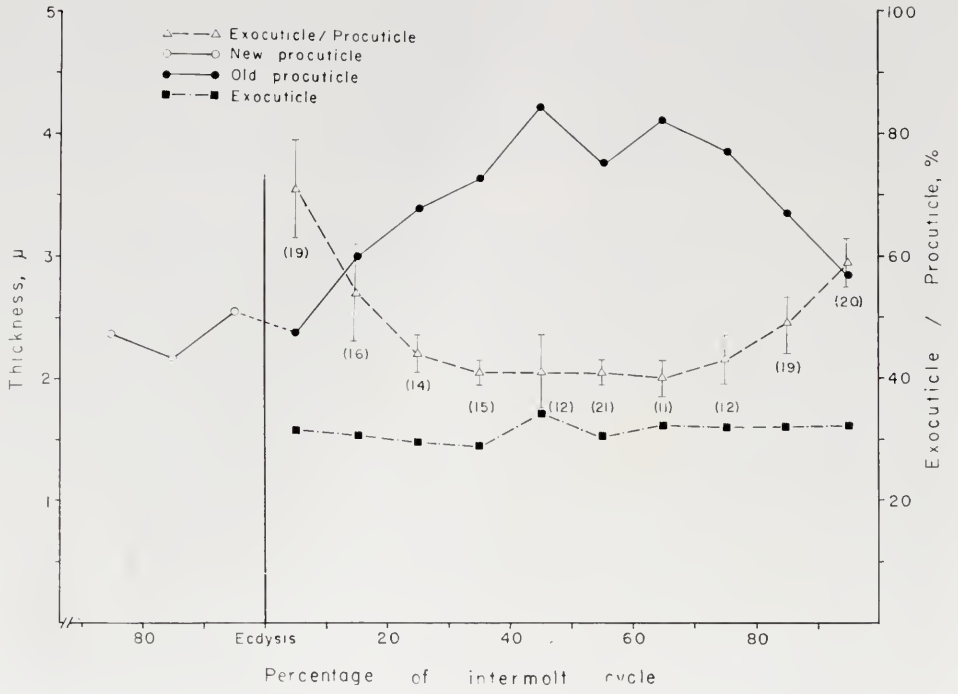


FIGURE 1. Thickness of the procuticle and of the exocuticle of *Balanus amphitrite* during the intermolt cycle. The stippled line shows the exocuticle expressed as a percentage of the corresponding procuticle. Each point is the mean of observations (number in parentheses) made for each 10 per cent interval of the intermolt cycle. The vertical bars represent 95% confidence limits.

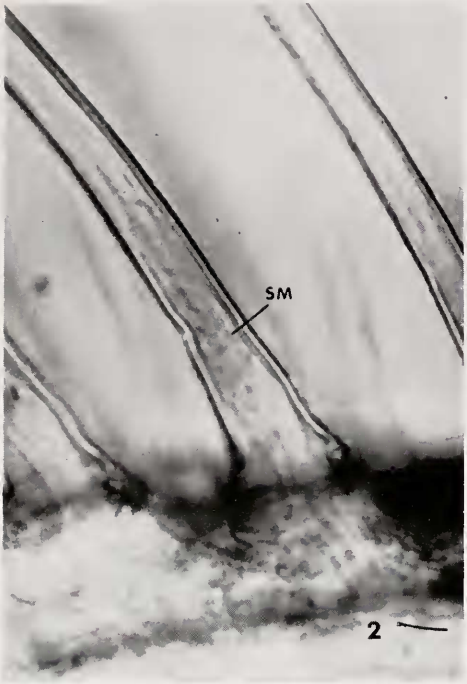
RESULTS

Criteria for the intermolt stages

Following the general outline of Drach (1939) we have divided the intermolt cycle of *B. amphitrite* into eight stages: A; B_1 and B_2; C; D_0, D_1, D_2, and D_3. The criteria by which these stages are identified are based upon (1) the overall form of the cirri, (2) the formation and the relative thicknesses of the exoskeletal layers, and (3) the progression of setogenesis.

The cirri. In a barnacle at rest the cirri normally assume a tightly curled form. Only in the first hours of postecdysis do the cirri deviate from this form and are tortuous, or twisted.

The exoskeleton. The exoskeleton of the rami as seen in the light microscope is composed of two layers (see Fig. 6). In live whole mounted rami and in glycerin jelly mounted rami the outer layer is dark green in color and the inner layer is light green. In sections of rami stained with Azan the outer layer is red and the inner layer blue. The outer and inner layers are assumed to be homologous (see Discussion) with the exocuticle and endocuticle, respectively, of the procuticle (the procuticle = exocuticle + endocuticle) of other crustaceans and the arthro-



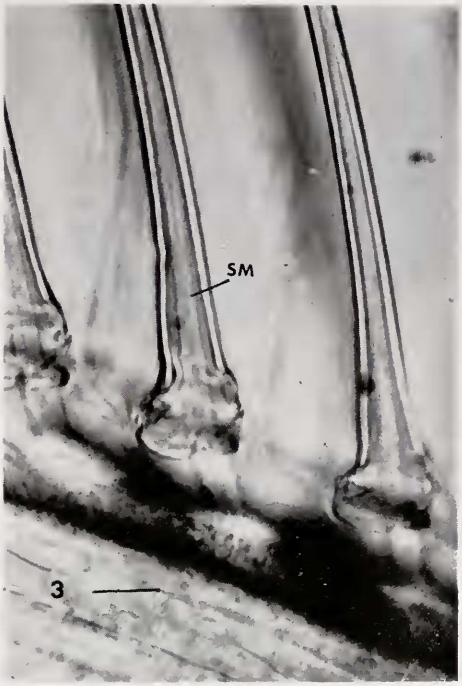


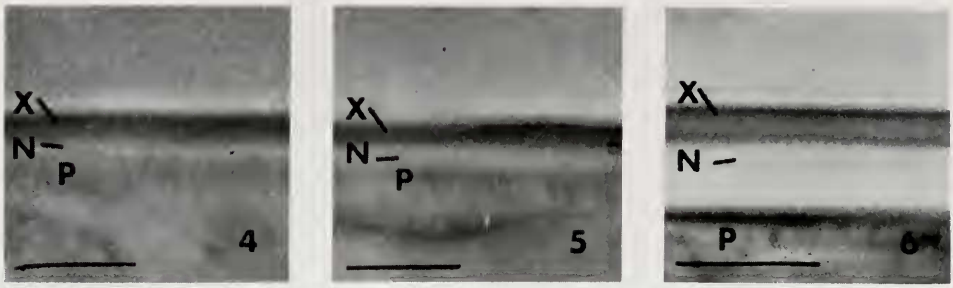
FIGURE 2. Setae of *Balanus amphitrite* in Stage C showing the setal matrix (SM) in the expanded state; ramus mounted in seawater; marker = 10 μ.

FIGURE 3. Setae of *Balanus amphitrite* in Stage D_0 showing the setal matrix (SM) in the contracted state; ramus mounted in seawater; marker = 10 μ.

pods in general (Hackman, 1971). These terms will be used throughout this paper.

As shown in Figure 1 a new procuticle is laid down in proecdysis before the animal has passed through 75 per cent of the intermolt cycle. The first presence of the new procuticle is difficult to distinguish from the granular epidermis. We have defined the new procuticle to be present when the segmental hinge on the posterior side of the ramus is present and when the layer making up the hinge is clear and nongranular in appearance (see Fig. 7). The new procuticle and the same procuticle immediately after ecdysis has a light green appearance and cannot be resolved into its component layers. The thickness of this procuticle, however, is greater than the thickness of the exocuticle (Fig. 1). We interpret this as meaning that the procuticle in this state is composed of the exocuticle and a small portion of the endocuticle but that they are not visibly differentiated, *i.e.*, the exocuticle is not sclerotized.

Within four hours after ecdysis the procuticle can be resolved into an exo- and endocuticle. The exocuticle then remains constant in thickness throughout the intermolt cycle while the procuticle increases in thickness in the first portion of the intermolt cycle and decreases in the last portion (Fig. 1). The changes in thickness of the procuticle thus reflect the changes which are occurring in the endo-



FIGURES 4–6. The exoskeleton on the posterior side of rami of *Balanus amphitrite* in post- and interecdysial stages: Figure 4 = Stage B_1; Figure 5 = Stage B_2; Figure 6 = Stage C; rami mounted in seawater; X = exocuticle, N = endocuticle, P = epidermis; markers = 5 μ.

cuticle. The stippled line in Figure 1 shows the thickness of the exocuticle expressed as a percentage of the thickness of the corresponding procuticle. This exocuticle percentage decreases from a high mean value of about 70 per cent in the first 10 per cent of the intermolt cycle to a mean of about 40 per cent between 30 and 70 per cent of the cycle, and then increases to close to a mean of 60 per cent in the last 10 per cent of the cycle. We have used the exocuticle percentage to define the limits of various intermolt stages since the values are indications as to whether the endocuticle is in a state of formation, steady state, or resorption.

Setogenesis. The setae of the rami are filled with a tissue matrix, the setal matrix. This matrix is continuous with a fibrous strand of tissue, the extrasetal matrix, which traverses the segment diagonally in the hypodermis. In post- and interecdysis the setal matrix is in an expanded state, *i.e.*, it completely fills the setae and has a loose appearance (Fig. 2). The first sign of proecdysis is a contraction of the setal matrix whereby a separation is formed between the setal exoskeleton and the setal matrix which now has a fibrous appearance (Fig. 3). A separation between the epidermis and the exoskeleton on the anterior side of the segment may also be found at this time.

Setogenesis then proceeds and the new setae are formed as invaginations running diagonally in the segment following the path of the extrasetal matrix (see Fig. 8). The invaginations are initiated anteriorly and proceed posteriorly in the segment. A complete invagination of the large, distal setae of a segment may run completely through its own segment and enter the next proximal segment. The tip of the new seta is formed within the basal portion of the old seta.

Criteria for the identification of the intermolt stages of *B. amphitrite* are then as follows:

Stage A (early postecdysis). The cirri are tortuous and the procuticle is thin and seemingly single-layered. There is no feeding activity or cirral beating but slow irregular movements of the cirri and opercular valves may be observed.

Stage B (late postecdysis). The cirri have regained their normal curled form. The setal matrix is expanded (Fig. 2). The procuticle is double-layered and the exocuticle percentage is more than 50 per cent. Stage B_1 is characterized by an exocuticle percentage of more than 65 per cent (Fig. 4) and Stage B_2 is characterized by an exocuticle percentage between 50 and 65 per cent (Fig. 5).

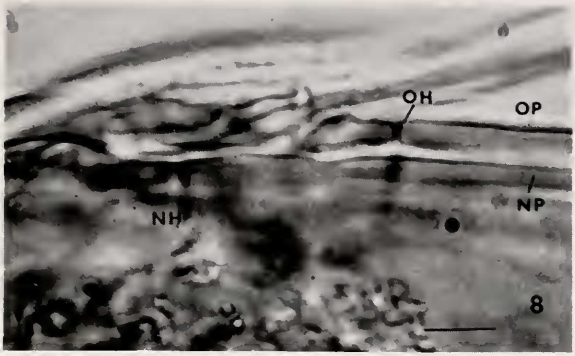


FIGURE 7. Posterior side of a ramus of *Balanus amphitrite* in Stage D_2 showing the new (NP) and old (OP) procuticles and the new (NH) and old (OH) segmental hinges; ramus mounted in glycerin jelly; marker = 5 μ.

Stage C (*intercedysis*). The setal matrix is expanded and the exocuticle percentage is 45 per cent or less (Fig. 6).

Stage D (*proecdysis*). Stage D_0 is characterized by the contraction of the setal matrix (Fig. 3), the rami otherwise having the appearance of Stage C. Stage D_1 is characterized by the presence of invaginations. During this stage the setal matrix retracts and the tip of the new seta is formed. Stage D_1 may be subdivided into two or more stages, the subdivisions being based on the increasing depth of the invaginations. Stage D_2 is characterized by the presence of a new procuticle on the posterior side of the segment (Fig. 7). The invaginated setae are more distinct and setules can be observed clearly on the tip of the new setae and faintly on the invaginated portion. The exocuticle percentage increases but is less than 60 per cent. Stage D_3 is characterized by an exocuticle percentage above

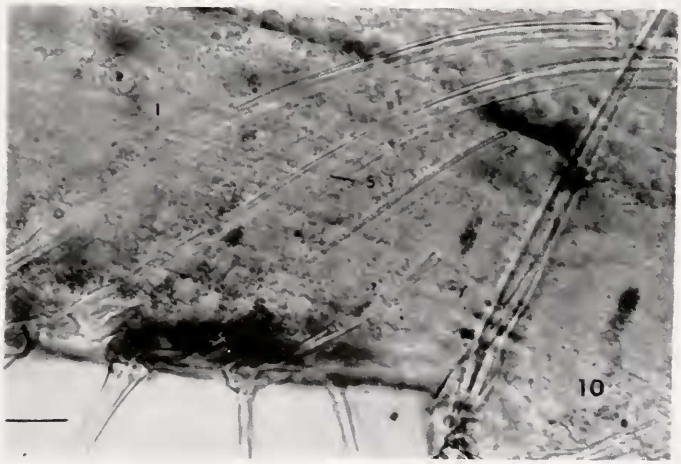


FIGURE 8. Invaginations (I) of the new setae of *Balanus amphitrite* in Stage D_3; ramus mounted in seawater; S = setules; marker = 100 μ.

TABLE I

Criteria for the intermolt stages of Balanus amphitrite

Intermolt stage	Cirri	Exocuticle percentage**	Setal matrix*	Invagination*	New procuticle**
A	Tortuous	100***	Expanded	Absent	Absent
B					
B_1	Normal	>65	Expanded	Absent	Absent
B_2	Normal	50–65	Expanded	Absent	Absent
C	Normal	<45	Expanded	Absent	Absent
D					
D_0	Normal	<45	Contracted	Absent	Absent
D_1	Normal	<60	Retraction or new tip	Faint	Absent
D_2	Normal	<60	New tip	Distinct	Present
D_3	Normal	>60	New tip	Obvious	Present

* Observed in seawater mounted rami.
** Observed in glycerin jelly mounted rami.
*** Seemingly single-layered.

60 per cent. The new procuticle is clearly seen and is thicker than the old exocuticle. The invaginated setae stand out brownish-yellow to the surrounding tissues (Fig. 8). Setules are clearly seen both on the tip and on the invaginated portion of the new setae.

A summarized outline of the intermolt stages and their criteria is presented in Table I.

Inter- and intraramial synchrony

The following studies were performed to determine whether the integumental changes in different segments of a ramus and the different rami of an animal proceed at an equal rate.

TABLE II

Thickness (μ) of the exocuticle (Exo) and procuticle (Pro) in segments of the inner (i) and outer (o) rami of the right 4th, 5th, and 6th cirri of a Stage C specimen of Balanus amphitrite. *The thickness of the exocuticle is also expressed as a percentage (Exo %) of the corresponding procuticle*

Ramus	5th Segment			13th Segment			20th Segment			Exo % mean ± S.E.
	Exo	Pro	Exo %	Exo	Pro	Exo %	Exo	Pro	Exo %	
6i	0.8	2.4	33	1.7	4.4	39	2.1	5.9	36	36 ± 1.4
6o	0.9	2.6	35	1.7	5.1	33	2.3	5.8	40	36 ± 1.4
5i	1.1	2.7	41	1.8	4.9	37	2.2	6.5	34	37 ± 1.7
5o	1.0	2.8	36	1.7	5.1	33	2.0	6.1	33	34 ± 0.8
4i	0.9	2.7	33	1.7	5.5	31	2.2	6.3	35	33 ± 0.9
4o	1.0	2.6	38	1.7	4.8	35	2.4	6.4	38	37 ± 0.8
Mean ± S.E.	0.95± 0.04	2.63± 0.05	36± 1.1	1.72± 0.02	4.97± 0.13	35± 1.1	2.20± 0.05	6.20± 0.11	36± 1.0	

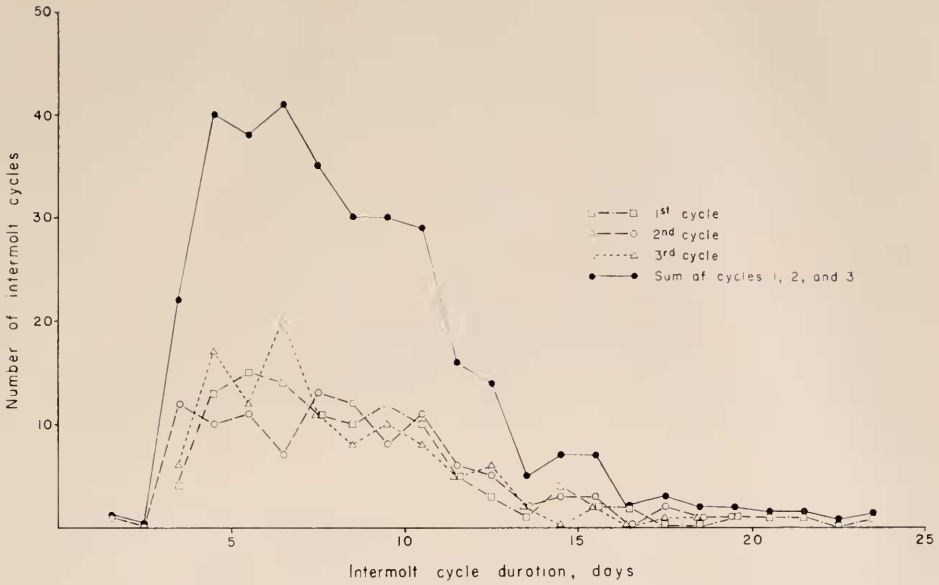


FIGURE 9. Frequency distribution of the durations of intermolt cycles of adult *Balanus amphitrite* kept in the laboratory at 23° C through three successive cycles.

The 5th, 13th, and 20th segments of the inner and outer rami of the right cirri of an animal were examined for variations in the thicknesses of the exoskeletal layers. The results (Table II) show that the exocuticle and the procuticle increase in thickness proximally within a ramus while the exocuticle percentages seem to be equal. The exocuticle percentages between the rami also seem to be equal.

The exocuticle percentages were calculated for the 13th segment of the inner rami of the right cirri of animals in different intermolt stages. Table III shows that the exocuticle percentages in a given animal had only small variations from ramus to ramus. Using the criteria in Table I this variation would not lead to different intermolt stages.

The integumental changes were characterized in the 5th, 13th, and 20th segments of the inner rami of the right cirri in nine animals in Stage D: two in D_0, three in D_1, three in D_2, and one in D_3. No differences were observed in the integumental changes either within or between the rami.

The above results show that setogenesis and the formation and resorption of the exoskeletal layers progress synchronously both inter- and intraramially.

Duration of the intermolt stages

The durations of the intermolt stages were calculated from the data derived from the animals used in developing the above criteria. The intermolt cycles of these animals ranged from 3 to 29 days (see also description of duration of intermolt cycle). Stage A was found to last less than four hours. Stage B lasted from

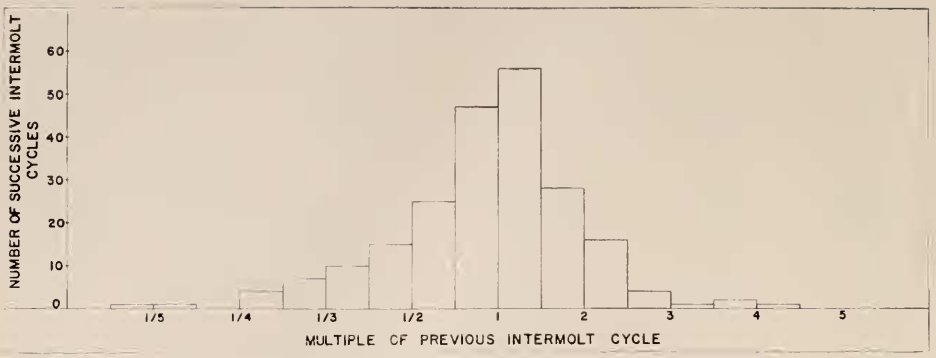


FIGURE 10. Frequency distribution of the relative durations of successive intermolt cycles of individual *Balanus amphitrite*. The duration of each intermolt cycle is calculated as a multiple of its previous cycle.

less than twelve hours to two days and Stage D lasted from one to five days. Stage C was found to be the most variable; it may outlast the other stages, or, in animals with short intermolt cycles, it may last less than one day. In terms of a percentage of an intermolt cycle, the stages also showed a large variation. Thus, Stage A made up less than 2 per cent; Stage B from 5 to 20 per cent; Stage C from 20 to 70 per cent; and Stage D from 20 to 40 per cent of the cycle.

Duration of the intermolt cycle

To analyze statistically the duration of the intermolt cycle of *B. amphitrite* 109 adult specimens were followed individually through three successive cycles (four ecdyses) from March to May, 1972. The durations of the intermolt cycles (Fig. 9) varied from 1.5 to 23.5 days. No significant difference was found between the frequency distributions of the three cycles (Kruskal-Wallis Test) showing that the population was stable throughout the experimental period. The three cycles when summed gave a mean duration of 8.3 days and a median of 7.6 days. Statistical tests for skewness and kurtosis showed that the distributions were significantly ($P < 0.001$) leptokurtic and positively skewed.

Each of the second and third intermolt cycles was calculated as a multiple of its previous cycle. These data (Fig. 10) show that the durations of successive intermolt cycles of individual animals also had a large variation. There was no correlation between the durations of successive intermolt cycles, the correlation coefficient being -0.08. Of the 218 successive cycles recorded, 107 had shorter and 108 had longer second cycles; 3 had identical intermolt cycle durations.

No correlation was found between the size (rostro-carinal diameters 5–10 mm) of the animal and the duration of the intermolt cycle. Also, ecdysis was not correlated with either the scotophase or photophase.

DISCUSSION

Two exoskeletal layers are visible in the cirri of *Balanus amphitrite* by light microscopy. Based on the following we assume the layers to be homologous with

TABLE III

Exocuticle thickness expressed as a percentage of the corresponding procuticle in the 13th segment of the inner rami of the right 4th, 5th, and 6th cirri of Balanus amphitrite *in various intermolt stages*

Intermolt stage	6th cirrus	5th cirrus	4th cirrus	Mean
B_1	68	68	70	69
B_1	67	72	74	71
B_1	69	68	65	67
B_2	57	51	59	56
C	36	35	35	35
C	35	39	38	37
C	44	42	44	43
C	31	30	35	32
C	34	33	32	33
D_1	33	31	33	32
D_1	49	46	47	47

the exocuticle and endocuticle of the arthropod procuticle. In the arthropods the exoskeleton, in general, is composed of three layers, a thin outermost epicuticle, the exocuticle, and the endocuticle (Hackman, 1971). By electron microscopy a three-layered exoskeleton is also found in *B. improvisus* (S. Koulish, Richmond College, Staten Island, New York, personal communication). The thickness of the outermost layer of this exoskeleton is below the resolving power of the light microscope so that by light microscopy only two layers would be visible. The sequence of formation and resorption of the exoskeletal layers in *B. amphitrite* during the intermolt cycle (Fig. 1) further supports the homology. The outer exoskeletal layer is discernible from early postecdysis and does not change in thickness during the intermolt cycle. This is consistent with the behavior of the arthropod exocuticle (Hackman, 1971). The main portion of the inner exoskeletal layer in *B. amphitrite* is secreted in postecdysis and resorbed in proecdysis. A small portion may also be secreted in proecdysis. This corresponds with the sequence of secretion and resorption of the arthropod endocuticle (Hackman, 1971).

The structural changes in the integument of *B. amphitrite* during an intermolt cycle are similar to those described for malacostracans (Passano, 1960; Yamaoka and Scheer, 1970). Drach's (1939, 1944) subdivision of the intermolt cycle is thus found to be applicable to cirripeds. In malacostracans post- and interecdysial stages are mainly characterized by the increasing rigidity of the exoskeleton, and the stages are identified by comparing the rigidity of various regions of the carapace (Drach and Tchernigovtzeff, 1967). Small size and the presence of the shell in cirripeds make palpation an impractical method for identifying the corresponding intermolt stages in these animals. Bocquet-Vedrine (1965) was unable to distinguish Stages B and C in *Elminius modestus*. We found, however, that in *B. amphitrite* these stages can be identified directly by microscopic examination of the exoskeleton of cirri mounted in glycerin jelly. The formation of the endocuticle is reflected in an increasing thickness of the procuticle and in a decreasing exocuticle percentage (Fig. 1). The criteria for the proecdysial stages in *B. amphitrite* are consistent with Drach's method (as noted by Bocquet, 1956, for stages D_1 and D_2 in

Chthamalus stellatus) except that resorption of the endocuticle is, again, observed directly rather than identified by palpation. The synchrony in the integumental changes allows the random use of the rami and of the segments within a ramus for intermolt staging. It is recommended, however, that the middle segments of the rami be used because of the conveniences of their size and the thickness of the exoskeleton.

It is difficult to obtain animals in Stage A because of its short duration. The best method of obtaining animals in this stage is to utilize animals within one hour of ecdysis. It should be noted, however, that the external presence of an exuvium attached to an animal is not, by itself, a suitable criterion for a recent ecdysis. We have observed that exuviae may remain attached to *B. amphitrite* in the laboratory for over three days after ecdysis. Likewise, we have observed *B. amphitrite* with exuviae attached in the field throughout the period of air exposure at low tide. Thus, the best method of obtaining newly molted animals is to make frequent observations of isolated individuals in Stage D_2 or D_3.

The frequency distribution of the durations of the intermolt cycles of a population of *B. amphitrite* has a large variability and is leptokurtic and positively skewed (Fig. 9). This type of distribution may be an intrinsic feature of the population since environmental factors were held constant and since successive intermolt cycles had similar distributions. It would appear that a minimum amount of time is needed for an animal to complete its exoskeleton following ecdysis and to prepare for its forthcoming ecdysis, with the majority of the animals in a population molting soon after the minimum time limitation. It is also interesting to note that despite the large variation in the durations of successive intermolt cycles of individual animals (Fig. 10), the variation in the population as a whole is such that the original distribution is maintained through successive cycles (Fig. 9). This phenomenon is also indicated by the nearly normal form of the frequency distribution of Figure 10. The large variability in the successive cycles of individual *B. amphitrite* does not support the notion of fast and slow molters as was proposed for *B. balanoides* (Patel and Crisp, 1961).

The large variability in the duration of the intermolt cycle and its stages in adult *B. amphitrite* stresses the inadequacies of the timed interval method as a procedure for intermolt staging in cirripeds. If the timed interval method was to be applied the animals could be in several different intermolt stages at any given interval after ecdysis. This could introduce large variation in the data and thus obscure any intermolt stage dependency. Moreover, the present method eliminates the requisite of a knowledge of the prehistory of the individual animal that is necessary for the timed interval method. This allows the use of animals chosen at random from either laboratory or field populations and thus broadly widens the scope of intermolt cycle related studies in cirripeds. The present method should be applicable to all thoracic cirripeds as long as the cirri are transparent enough to allow microscopic examination and as long as the proper exocuticle percentages are calculated for each species.

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Summary

The intermolt cycle of the barnacle *Balanus amphitrite* is divided into three postecdysial, one interecdysial, and four proecdysial stages based on integumental changes in the cirri. Stage A is characterized by a seemingly single-layered exoskeleton and tortuous cirri. Stages B_1, B_2, and C are characterized by the increasing thickness of the endocuticle. Stage D_0, D_1, D_2, and D_3 are characterized by the progression of setogenesis, formation of the new exoskeleton, and resorption of the old endocuticle. The durations of the intermolt stages have a wide variability. The integumental changes both within and between the rami of an animal progress synchronously. The criteria allows the use of live, intact animals taken at random from laboratory or field populations. The method is assumed to be applicable to all thoracic cirripeds as long as the exoskeleton is transparent enough to allow microscopic examination.

The duration of the intermolt cycles of adult specimens of *B. amphitrite* varied from 1.5 to 23.5 days under constant conditions (23° C, 30‰, LD 12:12). The distributions of the cycles are significantly ($P < 0.001$) leptokurtic and positively skewed. There is no correlation between the durations of successive intermolt cycles of individual animals.

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